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Danio annulosus, a new species of chain *Danio* from the Shuvolong Falls in Bangladesh (Teleostei: Cyprinidae: Danioninae)

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Abstract

Danio annulosus, new species, is described from a small pool below the Shuvolong Falls in the Kaptai Lake system in Bangladesh. It shares with chain danios (*D. assamila, D. dangila, D. catenatus, D. concatenatus*, and *D. sysphigmatus*) a colour pattern consisting of series of dark rings with light interspaces along the side, complete lateral line, 14 circumpeduncular scales, a produced first ray in the pectoral fin, and a black humeral spot. It differs from other chain danios in possessing much shorter pectoral and pelvic fins, and a humeral spot that is slightly wider than deep instead of round or deeper than wide. The mitochondrial cytochrome *c* oxidase subunit I (COI) sequence separates *D. annulosus* from the most similar species, *D. catenatus* by a *p*-distance of 3.4%. Although recorded from only a single locality, *Danio annulosus* is expected to have a wider distribution in the Karnafuli River drainage.

Key words: DNA barcode, freshwater, morphometrics, phylogeny, taxonomy

Introduction

The southern Asian cyprinid fish genus *Danio* Hamilton comprises 24 valid species, most of which are native to India, Bangladesh, and Myanmar, although some species are also found in Pakistan, Laos, Thailand, southern China, Cambodia, Viet Nam, Malaysia, and the Indonesian island of Sumatra (Fang Kullander, 2001; Kullander & Britz, 2015). They are small fishes, between about 20 and 90 mm standard length, with distinctive colour patterns providing both important diagnostic characters and making them attractive ornamental fishes (Collins *et al.*, 2012; Fang Kullander, 2001; Kullander, 2015). The genus has gained particular interest as it includes the zebrafish *Danio rerio* (Hamilton), which is an important vertebrate model organism for biomedical research (Parichy, 2015). Several recent studies have analysed the interrelationships (e.g., Fang, 2003; Tang *et al.*, 2010) and species taxonomy of the genus (e.g., Kullander, 2015; Kullander & Britz, 2015), but much of the diversity remains undescribed.

Kullander (2015) recognized a distinctive group of five species of *Danio* characterized by the flank colour pattern, consisting of series of dark rings enclosing light centres somewhat similar to chains, which he named informally as chain danios: *D, assamila* Kullander, *D. catenatus* Kullander, *D. concatenatus* Kullander, *D. dangila* (Hamilton), and *D. sysphigmatus* Kullander. Chain danios are also distinctive in having a complete lateral line and 14 circumpeduncular scales, shared with *D. meghalayensis* Sen & Dey, *D. absconditus* Kullander & Britz, and *D. feegradei* Hora, but different from other *Danio* in which the lateral line is absent or abbreviated, and circumpeduncular scales vary between 10 and 12 (Kullander, 2015). Chain danios share with *D. feegradei* a relatively large size, 60–88 mm SL (Kullander, 2015), contrasting with the majority of *Danio*, which reach only between about 20 and 35 mm SL (Kullander & Fang, 2009).

In a survey of Bangladeshi freshwater fishes specifically for building a DNA barcode reference library, we encountered a species of chain *Danio* similar to *D. catenatus* Kullander and *D. concatenatus* Kullander from western Myanmar, but distinctive in mitochondrial DNA, details of the colour pattern, and with a shorter pectoral fin. This species was collected from the foot of a spectacular waterfall in the Chittagong Hills Tract draining to the man-made Kaptai Lake, which remains the only known locality for the species. The present paper provides a

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formal description of this new species and an assessment of its relationships and possible threat status, and also establishes its DNA barcode based on the holotype.

Material and methods

Specimens are kept in the following collections: NRM, Swedish Museum of Natural History, Stockholm; DU, Zoology Department, University of Dhaka, Dhaka. Some comparative material is kept in the Natural History Museum, London (BMNH). Measurements were taken with digital callipers to a precision of 0.1 mm. Counts and measurements were made according to Fang (1997) and Kullander (2015). Colour pattern terminology follows Fang (1998) with modifications for special markings as explained by Kullander (2015). Horizontal stripes are identified by alphanumeric annotations. Fin-ray and vertebral counts were taken from X-radiographs made with a Philips MG-105 low voltage X-ray unit and Kodak EDR2 plates. Abdominal vertebrae counts include the Weberian apparatus (assumed to contain four centra). Statistics were calculated using SYSTAT v. 13 (Systat Software, 2009), except that the principal component analysis (PCA) of measurements was made using a separate procedure for component shearing, partialling out multivariate-size residues from the second and further components as described by Humphries et *al*. (1981). The PCA was made with log-transformed measurement data to tenth of a millimetre in a covariance matrix, and without rotation. An asterisk (*) marks counts from the holotype.

Species delimitation was tested using the standard mitochondrial 5' cytochrome oxidase subunit I (COI, also known as mt-CO1 or COX1) DNA barcoding fragment, as suggested by the Barcode of Life consortium (Steinke & Hanner, 2011).

DNA was extracted using a Thermo Scientific™ KingFisher™ Duo (Thermo Fisher Scientific) fully automated liquid-handling instrument, with the Thermo Scientific KingFisher Cell and Tissue DNA Kit (Thermo Fisher Scientific) and recommended protocol. The COI fragment was amplified using the fish barcoding primers Fish-F1 and Fish-R1 (Ward et al. 2005). PCR were performed with the puReTaq Ready-To-Go PCR kit (Amersham Biosciences AB, Uppsala, Sweden). PCR cycling: 94°C 4min; 35 * (94°C 30s; 52°C 30s; 72°C 30s); 72°C 8min). PCR products were checked on agarose gel, and purified by adding 5µL of a mix consisting of 20% Exonuclease I (EXO) and 80% FastAP Thermosensitive Alkaline Phosphatase (Fermentas/Thermo Fischer Scientific, Gothenburg, Sweden) to each 25 µL PCR reaction, incubated at 37°C for 30 minutes, then heated to 80°C for 15 minutes.

Sequencing of both strands of all fragments was carried out by Macrogen Europe (Amstelveen, Holland). All sequences were proofread and assembled using the software Geneious R8 (Kearse *et al*., 2012).

651 basepairs from the 5' end of the COI gene of three individuals of *Danio annulosus*, including the holotype, and all other available species of chain danios were sequenced. Additional sequences were obtained from GenBank and the supplementary dataset S1 in Collins *et al.* (2012). A total of 40 sequences representing 14 species were aligned using the software Geneious with the MUSCLE (Edgar, 2004) plug-in, and edges were manually trimmed to a total alignment length of 651 basepairs. DNA sequences and vouchers are listed in Table 1.

Sequences prefixed with RC were taken from Collins *et al*. (2012). They submitted their COI sequences to the Barcode of Life Database (BOLD). However, it turns out that they, probably by mistake have uploaded their Rhodopsin data (labelled as Rhodopsin) instead of the COI data to BOLD. As a result, Collins *et al*. (2012) COI sequences are available neither from BOLD nor GenBank, and they have no GenBank accession number. Although the COI sequences in Collins *et al.* (2012) are not published in GenBank, are based on aquarium specimens, and presently are only available as supplementary information with Collins *et al.* (2012: their Dataset S1, doi:10.1371/journal.pone.0028381.s004), we elected to include them, as they correspond to those identified from wild material. Species identifications based on photos in BOLD had been made previously by Kullander (2015).

Phylogenetic analysis was performed using the software MrBayes v3.3 (Huelsenbeck & Ronquist, 2001; Ronquist *et al.*, 2014). Data were partitioned according to codon position (first, second, third) and parameters estimated separately for each partition. *Devario* was designated outgroup in all analyses. The GTR + Γ + I model was used as suggested by ModelTest (Posada & Crandall, 1998). Samples were taken every 1000 generations, and the first 25% of samples were discarded as 'burn-in'. The analysis was run for ten million generations. Convergence was confirmed with MrBayes (average standard deviation of split frequencies <0.004), and the software Tracer v1.6 (Rambaut *et al.*, 2014) (effective sample size = 8612). Unless otherwise stated all distances are uncorrected pairwise *p*-distances, as recommended by Srivathsan & Meier (2011). The software Geneious with the plug-in Species Delimitation (Masters *et al.*, 2011) was used to obtain "corrected" estimates of pairwise *p*-distance and for calculating the probability of reciprocal monophyly under a model of random coalescence.

Comparative material. Specimens used by Kullander (2015) and Kullander & Britz (2015).

TABLE 1. GenBank accession numbers and voucher catalogue numbers for COI sequences used in this paper.

GenBank#	Voucher#	Species
HM224143	NO DATA	*Danio albolineatus*
KT199744	DU 50001	*Danio annulosus*
KT199745	DU 60001	*Danio annulosus*
KT199746	NRM 66255	*Danio annulosus*
KT199747	NRM 42645	*Danio assamila*
KT199748	NRM 51440	*Danio assamila*
KT199749	NRM 47423	*Danio assamila*
KT199750	NRM 47422	*Danio assamila*
MISSING	RC0343	*Danio assamila*
KT199751	NRM 41676	*Danio catenatus*
MISSING	RC0561	*Danio concatenatus*
MISSING	RC0563	*Danio concatenatus*
MISSING	RC0564	*Danio concatenatus*
KT199752	NRM 49818	*Danio dangila*
KT199753	NRM 50197	*Danio dangila*
HM224144	NO DATA	*Danio dangila*
NC_015525	NO DATA	*Danio dangila*
MISSING	RC0122	*Danio dangila*
MISSING	RC0123	*Danio dangila*
MISSING	RC0344	*Danio dangila*
MISSING	RC0345	*Danio dangila*
MISSING	RC0346	*Danio dangila*
MISSING	RC0347	*Danio dangila*
MISSING	RC0348	*Danio dangila*
EF452861	NO DATA	*Danio feegradei*
HM224146	NO DATA	*Danio feegradei*
HM224148	NO DATA	*Danio kyathit*
HM224149	NO DATA	*Danio margaritatus*
KT199754	NRM 53806	*Danio meghalayensis*
KT199755	NRM 53967	*Danio meghalayensis*
MISSING	RC0565	*Danio meghalayensis*
MISSING	RC0566	*Danio meghalayensis*
MISSING	RC0567	*Danio meghalayensis*
MISSING	RC0568	*Danio meghalayensis*
KT199756	NRM 57107	*Danio sysphigmatus*
MISSING	RC0560	*Danio sysphigmatus*
MISSING	RC0562	*Danio sysphigmatus*
JF915580	YGN511	*Danio tinwini*
JX260863	CEGA02	*Devario devario*
JX983285	NF675	*Devario malabaricus*

***Danio annulosus,* new species**

(Figs. 1a–b)

Holotype. DU 50001, adult, probable female, 71.5 mm SL; Bangladesh: Chittagong Division, Rangamati District, Borokal, pool at bottom of Shuvolong Waterfall. M. M. Rahman, S. O. Kullander, M. Norén, A. Obaida, M. Hossain and M. Rashid, 28 Nov 2014.

Paratypes. NRM 67114, 4, 50.1–68.2 mm SL; NRM 66255, 1, 45.8 mm SL; DU 60001, 4, 41.8–50.7 mm SL. Same data as holotype.

Diagnosis. Distinguished from all other species of *Danio* except *D. assamila, D. dangila, D. catenatus, D. concatenatus,* and *D. sysphigmatus* by produced first ray in pectoral and pelvic fins, large cleithral spot, and pattern of dark rings enclosing light interspaces on the side. Distinguished from those species by slightly horizontally extended cleithral spot (vs. vertically extended in *D. dangila,* round in the other species), anterior interstripe Ia usually present (vs. absent in *D. dangila* and *D. catenatus*), rings in series along side elongate, width of each dark ring of about same as diameter of light centre or wider (vs. elongate, with narrower dark ring in *D. sysphigmatus*, round or slightly elongate and with dark ring about as wide as light centre in other species), ring pattern usually not extending onto caudal peduncle (vs. present on part of caudal peduncle in *D. dangila, D. catenatus* and *D. concatenatus*), lateral-line scale count 34–35, (vs. 35–38 in *D. sysphigmatus*), pectoral fin barely or not reaching to base of pelvic fin, unbranched leading ray only slightly longer than remaining rays (vs. leading ray prominently elongated, reaching to base of unbranched pelvic-fin ray or beyond) (Figs. 1–2), pelvic fin without produced unbranched ray, not reaching to vent (vs. with unbranched ray distinctly longer than the rest, and reaching to vent or bases of anterior anal-fin rays) (Figs. 1–2).

Description. General body features and pigmentation are illustrated in Fig. 1. Measurement data are summarized in Table 2.

Body compressed, moderately elongate to moderately deep, no sexual dimorphism evident. Head compressed, about as wide as deep. Snout short, rounded in lateral view, length about equal to eye diameter. Mouth terminal, oblique in profile, lower jaw slightly projecting. Small bony knob at dentary symphysis, fitting into notch in upper jaw. Maxilla reaching to vertical from anterior margin of orbit. Lower jaw ending anteriorly at about middle of eye. Lower jaw with pointed anterior lateral lobe. Tubercles absent from lower jaw or present in a row along lateral margin of dentary and/or a few small tubercles anteriorly. Rostral barbel reaching little past preopercle, at most to vertical at middle of opercle. Maxillary barbel long, reaching to middle of base of pectoral fin or slightly posterior to base of pectoral fin.

Lateral line complete, comprising 34* (1), 35 (9) scales; descending anteriorly for about 6 scales, from some of which pores are absent, posteriorly paralleling ventral profile. Median predorsal scales 16 (1), 17* (7), 18 (2). Scales in transverse series from dorsal-fin origin to pelvic-fin origin ½6+1+1½* (10); scales below lateral line much smaller than above. Prepelvic scales about 14–17 along prepelvic midline; pelvic-fin bases covered by overlapping scales arranged in three rows. Circumpeduncular scale rows 14* (10). Pelvic axillary scale present. A row of scales along anal-fin base.

Dorsal-fin rays ii.9½ (2), ii.10½ (6), ii.11½* (2); anal-fin rays iii.13½ (1), iii.14½* (6), iii.15½ (3); pectoral-fin rays i.12 (4), i.13 (6), including 1–2 minute unbranched ventral rays; pelvic-fin rays i.7* (10). Principal caudal-fin rays 10+9 (7); procurrent caudal-fin rays dorsally 5 (2), 6 (2) 7 (2), ventrally 5 (1), 6 (4). Dorsal fin inserted at highest point of dorsum, slightly posterior to half distance from snout tip to caudal-fin base, distinctly anterior to vertical from anal-fin origin. Pectoral-fin insertion at about vertical through posterior margin of opercle; branched rays forming slightly rounded or subtruncate margin, leading unbranched ray produced beyond rest of fin, just reaching base of unbranched pelvic-fin ray (68.2 mm specimen only, Fig. 2) or shorter. Tubercles present on pectoral fin on 2nd to 4th or 5th branched ray in three paratypes (50.1, 50.7, 68.2 mm SL). Pectoral-fin axial lobe well developed. Pelvic-fin origin situated slightly anterior to midbody, well anterior to dorsal-fin origin. Pelvic-fin margin slightly rounded or truncate, leading unbranched ray only slightly longer than remainder, not reaching vent. Caudal fin slightly emarginate, lobes about equal, lobe tips subacuminate or rounded.

Vertebrae 17+18=35 (1), 17+19=36 (4), 18+17=35 (1), 18+18=36 (1); predorsal vertebrae 14 (7); vertebrae contained within caudal peduncle 5 (1), 6 (4), 7 (2). Ceratobranchial 5 tooth formula 2,4,5-5,4,2 (NRM 66255).

FIGURE 1. *Danio annulosus*: **a**, holotype, DU 50001, 71.5 mm SL, probable female; preserved in 95 % ethanol; **b,** paratype, NRM 67114, male, 68.2 mm SL, fixed in formalin; **c**, paratype, NRM 67114, probable female, 56.0 mm SL, fixed in formalin. All from Bangladesh, Kaptai Lake, Shuvolong Falls.

Colouration in preservative. Description based on specimens fixed in formalin and transferred to ethanol (Fig. 1b, c). Specimens initially preserved in 95% ethanol similar but with white semi-transparent overlay (Fig. 1a). Ground colour fawn or dull yellow, ventral midline white, head pale grey. Markings absent from head and venter, except slight concentration of dark pigment adjacent to lower margin of orbit and centre of opercle. Dorsal midline anterior and posterior to dorsal fin marked by narrow dark-brown stripe. Dark brown or grey stripes P, P+1, P+2, P-1, P-2; P+3 stripe absent. Cleithral spot black, slightly longer than deep (depth 70–96% of length, mean 77%, N=10), about as large as pupil. P+ stripe expressed as small round spots and short lines in irregular row from above upper end of gill cleft to root of caudal peduncle. P+1 and P stripes distinct on caudal peduncle, where forming straight parallel stripes, ending on scaled base of caudal fin; anteriorly anastomosing, contributing to a row of 4–10 contiguous elongate dark rings enclosing interstripe I. P-1 stripe distinct, narrow posteriorly, paralleling posterior parts of P and P+1 stripes, but ending in upward slant on basal scales of caudal fin; for most of extent anastomosing with lower branch of P stripe to form 4–7 large elongate rings fragmenting interstripe I-1; ring pattern irregular anteriorly on side with occasional absent ring parts. Narrow interstripe Ia inserted between dorsal and ventral rows of rings, expressed as subcontinuous stripe or irregular circle centres, extending from below cleithral spot to about vertical from anterior anal-fin rays. P-2 stripe indistinct, undulating, narrow, originating at pectoral-fin base, ending above posterior part of anal-fin base. Dorsal fin hyaline basally; dark interradial spots proximally forming dark stripe close to base; distally wide grey zone margined with darker grey on about middle of fin (D stripe); wide distal margin white. Anal fin hyaline basally; dark interradial spots forming dark stripe at base; dark interradial spots forming dark stripe (A-1 stripe) marking about proximal third of width of fin; outer two thirds of fin pale grey, margined by darker grey A stripe, distal margin of fin white anteriorly. Caudal fin dusky, lighter along middle, with distinct dark stripes or series of spots continuing stripes P+1, P and P-1. Sexual dimorphism absent in colour pattern.

TABLE 2. Morphometry of *Danio annulosus.* Measurements are in per cent of standard length, except for standard length (in mm). SD, standard deviation; r, Pearson's correlation coefficient. Linear regression against SL calculated from measurements in mm. HT= Holotype.

	N	HT	min	max	mean	SD	a	b	r
Standard length (mm)	10	71.5	41.8	71.5	53.2	9.8			
Body depth	10	26.2	24.3	28.0	25.5	1.0	-2.242	0.298	0.986
Head length	10	22.5	21.7	23.4	22.7	0.4	0.919	0.209	0.995
Snout length	10	6.7	5.9	6.8	6.5	0.3	0.337	0.059	0.962
Head depth	10	14.7	13.4	15.8	14.8	0.8	0.233	0.144	0.960
Head width	10	12.2	11.7	13.0	12.5	0.4	0.340	0.118	0.986
Upper jaw length	10	9.0	8.2	9.7	9.2	0.4	0.921	0.074	0.969
Lower jaw length	10	10.9	9.7	11.8	11.0	0.6	1.296	0.085	0.946
Orbital diameter	10	6.2	6.2	7.7	7.0	0.5	1.359	0.044	0.965
Interorbital width	10	10.1	9.8	10.5	10.2	0.2	0.366	0.095	0.994
Caudal peduncle length	10	15.2	13.5	16.5	15.3	0.9	1.822	0.118	0.952
Caudal peduncle depth	10	12.4	10.9	13.9	12.0	0.9	-1.847	0.155	0.969
Dorsal-fin base length	10	17.2	15.4	18.0	16.7	0.9	-1.165	0.189	0.972
Anal-fin base length	10	22.0	19.4	23.3	21.5	1.1	-1.948	0.253	0.981
Predorsal length	10	59.4	57.6	61.4	59.7	1.0	1.881	0.561	0.996
Preanal length	10	65.2	64.5	66.9	65.5	0.8	0.017	0.655	0.998
Prepelvic length	10	46.9	44.9	48.3	46.6	0.9	1.426	0.439	0.994
Pectoral-fin length	10	19.4	19.4	25.1	23.2	1.6	2.949	0.175	0.895
Pelvic-fin length	10	13.8	12.8	16.1	14.5	1.0	-0.697	0.158	0.946
Rostral barbel length	10	13.6	13.0	18.0	15.3	1.4	2.743	0.100	0.826
Maxillary barbel length	10	20.0	20.0	24.6	22.9	1.5	3.759	0.156	0.928

Colouration in life. Freshly captured specimens had same colour pattern as preserved specimens. Dark stripes (including circles) were blue and interspaces (including circle centres) were orange. A distinct orange spot anterior to black cleithral spot. Some of orange colour remaining for some time in preserved specimens (Fig. 1c).

Geographical distribution and habitat (Figs. 3–5). *Danio annulosus* is so far known only from the type locality at the foot of a waterfall draining into the Kaptai Lake. The Kaptai Lake is a reservoir formed by the damming of the Karnafuli River, completed in 1962. In the wet season, the fall has a height of about 100 m and falls directly into the lake. At the time of collecting, the lake level was low: the waterfall had almost ceased and only some dripple moistened the rock. Water was retained in a small pool at the foot of the fall, at most about 40 cm deep, holding some pieces of rock but devoid of vegetation. Specimens were seined from the deeper shadowed area of the pool along with numerous specimens of shrimp, snails, and a small species of *Garra* Hamilton. The pool drained to the lake by a small stream, only about 1 cm deep. Seining in the lake at the mouth of the stream yielded *Esomus danrica* (Hamilton)*, Rasbora daniconius* (Hamilton) (Cyprinidae), *Dermogenys burmanica* Mukerji (Zenarchopteridae)*, Aplocheilus panchax* (Hamilton) (Aplocheilidae), and *Trichogaster lalius* (Hamilton) (Osphronemidae), which are ubiquitous species in Bangladeshi freshwaters.

Etymology. The species name, *annulosus*, is a Neolatin adjective meaning ringed, inspired by the colour pattern on the side, which resembles series of dark rings.

FIGURE 2. Ventral aspect of **a**, *Danio annulosus,* NRM 67114, 68.2 mm SL, with unusually long pectoral fins; **b**, *Danio concatenatus* (BMNH 2011.3.24.50, 61.1 mm SL), to show difference in produced pectoral and pelvic fins between *D. annulosus* (pectoral fin reaching base of pelvic fin or shorter; pelvic fin not reaching vent) and other chain danios (pectoral fin reaching beyond base of pelvic fin; pelvic fin reaching vent).

Comparative morphometrics. *Danio annulosus* has slightly shorter pectoral and pelvic fins (Fig. 6 a–b, shorter barbels (Fig. 6 c), and less deep head and body compared to other chain danios. Principal Component analysis separates *D. annulosus* from all other *Danio* with complete lateral line, except that one outlier (NRM 67114, 68.2 mm) clusters with other danios (Fig. 6 d; Table 3). The highest loadings are in head and body depth, dorsal-fin base length, and pectoral- and pelvic-fin lengths (Table 3).

Genetic distance analysis. The sequences ("barcodes") of all three sequenced specimens of *D. annulosus* were identical (pairwise *p*-distance = 0%). In our dataset *D. annulosus* was most similar to *D. catenatus* (3.4% pairwise *p*-distance). A BLAST (Altschul *et al*., 1990) nucleotide search of the GenBank non-redundant database (*nr*) found no exact matches. The closest matches were *D. dangila* (8% pairwise p-distance) and *Danio* cf. *dangila* (8%), followed by an unseparated group of different species of *Danio* (≥11%).

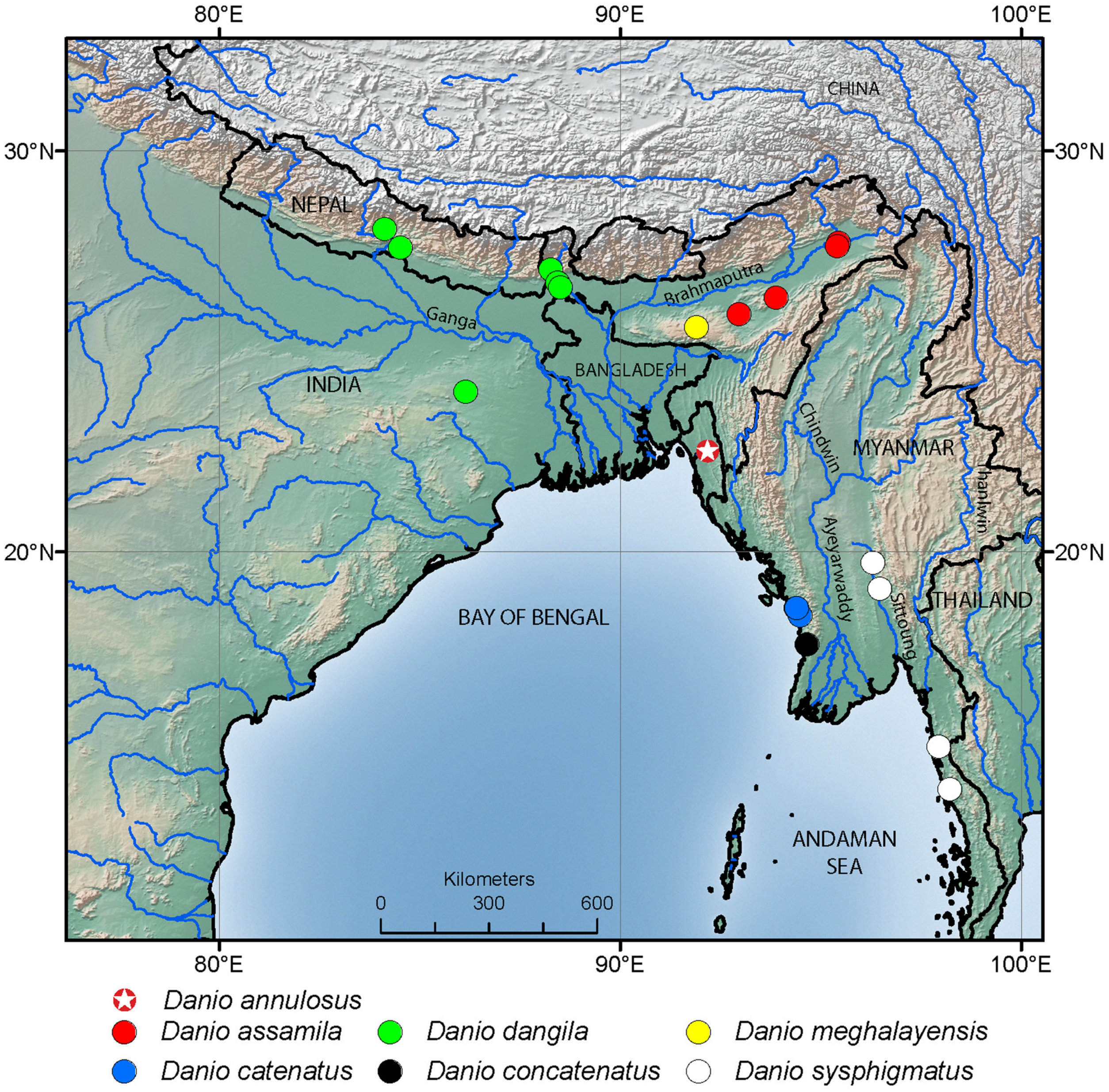

FIGURE 3. Collecting sites of chain danios and *Danio meghalayensis*; modified from Kullander (2015), with *Danio annulosus* added.

Entering the *D. annulosus* barcode into the identification tool at the Barcode of Life database (BOLD) portal (www.boldsystems.org) and searching for a match at the "species" level found no matches. A search of "all barcode records on BOLD" found that the five closest matches in BOLD were *Danio* aff. *dangila* (= *D. sysphigmatus* and *D. concatenatus*) (6.6% pairwise *p*-distance), *D. dangila* (8.1–8.5%), *Danio* cf. *dangila* (= *D. assamila*) (8.9%), *D. meghalayensis* (9.7%) and *D. erythromicron* (Annandale) (11.5%).

Phylogenetic analysis. The result of the phylogenetic analysis is summarized in Figure 7. *Danio* is monophyletic (Bayesian posterior probability, BPP 1.00). Within *Danio*, there is a trichotomy comprising the monophyletic chain danios (BPP 0.89), and two clades of non-chain danios. The first clade is *D. margaritatus* (Roberts) + *D. feegradei* (BPP 0.79), in the other clade (BPP 0.87) *D. albolineatus* (Blyth) is the sister group to *D. kyathit* Fang + *D. tinwini* Kullander & Fang (BPP 0.69). The chain danios comprise two clades. In the first clade (BPP 0.66), *D. dangila* is the sister of *D. meghalayensis* + *D. assamila* (BPP 0.99). In the second clade (BPP 0.99) *D. concatenatus* + *D. sysphigmatus* (BPP 1.00) are sister group to a clade comprising *D. catenatus* + *D. annulosus* (BPP 1.00).

FIGURE 4. Type locality of *Danio annulosus,* at foot of Shuvolong Falls, at this time almost expired (28 November 2014).

FIGURE 5. Type locality of *Danio annulosus*, close up of pool at foot of the waterfall where the type series was sampled.

Discussion

The chain danios make up a distinctive group by the shared colour pattern consisting of series of dark rings along the side, and the elongate leading rays in the pectoral and pelvic fins. The species described so far are distinguished mainly by slight differences in the colour pattern. *Danio annulosus* shares colour pattern, complete lateral line, and 14 circumpeduncular scales with other chain danios, and also agrees in morphometrics and meristics. The barbels are slightly shorter in *D. annulosus.* The pectoral fin has a slightly produced leading ray, but it is not as conspicuously long as in the other chain danios, and in the pelvic fin the leading ray is longer than the rest, but not produced. The difference in fin shape is reflected both in the proportional measurements, and in the shorter extension of the pectoral fin, barely or not reaching the insertion of the pelvic fin, and the pelvic fin not reaching to the vent. The 68.2 mm male of *D. annulosus* has relatively longer pectoral and pelvic fins than the other specimens including the holotype (71.5 mm female), but still shorter than in other chain danios at corresponding standard length. This condition may reflect sexual dimorphism in fin length or shrinkage in the holotype, which was preserved in 95% ethanol.

In the colour pattern, the slightly elongate cleithral spot is distinct from the vertically extended spot in *D. dangila* and the round spot in remaining species of chain danios. There is no clear autapomorphy in the pattern of rings. The presence of two relatively regular series of rings along the middle of the side and the more or less continuous interstripe Ia are similar to *D. concatenatus* and *D. sysphigmatus*. The presence of rings on the caudal peduncle varies, as in some other chain danios, as does the relative width of dark ring and light ring centre. The somewhat elongate rings are more similar to *D. assamila* and *D. sysphigmatus.*

The phylogenetic analysis, summarized in Fig. 7, shows that *D. annulosus* is monophyletic and distinct, and that the closest known relative is *D. catenatus* from south-western Myanmar. All genetic analyses support viewing *D. annulosus* as a distinct species.

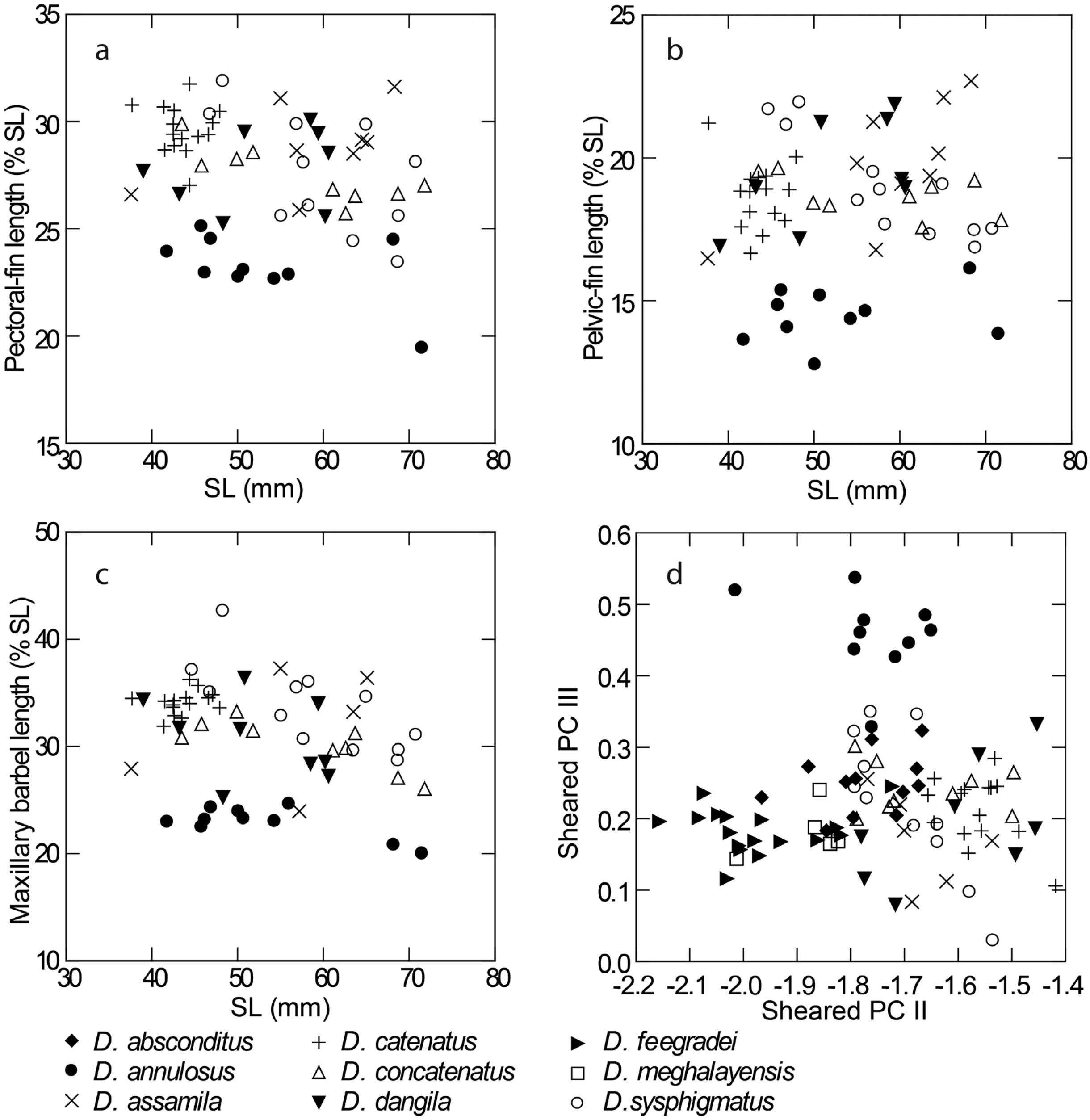

FIGURE 6. Comparative morphometry of *Danio annulosus.* **a–c,** pectoral-fin, pelvic-fin and maxillary barbel lengths as percent of SL, plotted against SL, in chain danios; d, Plot of scores of sheared Principal Component 3 against sheared Principal Component 2 from pooled measurements of all species of *Danio* with complete lateral line.

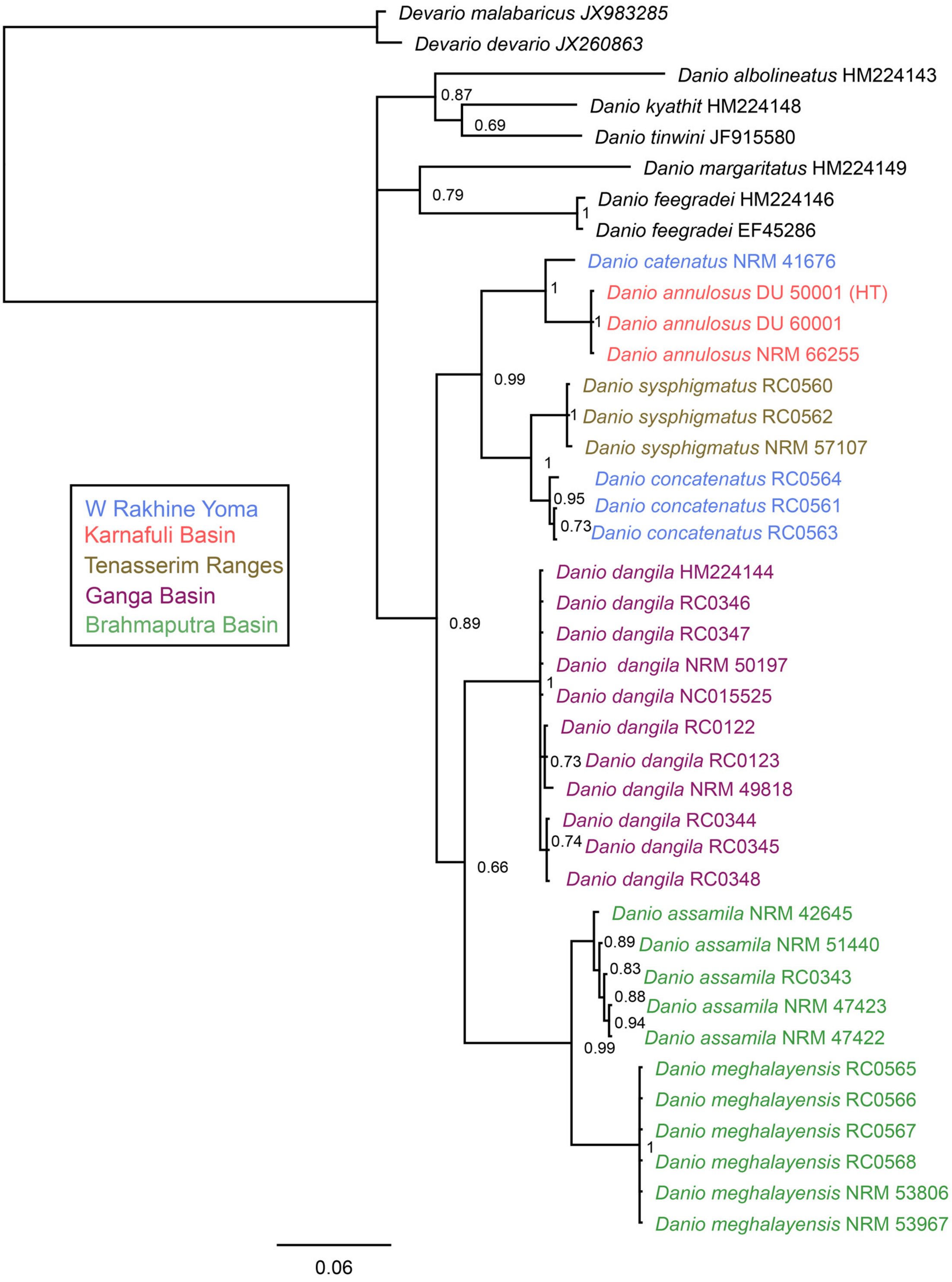

FIGURE 7. Bayesian majority-rule tree from analysis of mitochondrial COI DNA data from danionine outgroup, chain danios and *D. meghalayensis*. Values at nodes indicate Bayesian posterior probability estimates. Branch lengths are proportional to number of expected substitutions per site. HT indicates holotype of *Danio annulosus.* Sequences labeled with DU or NRM museum catalog number, except sequences from Collins *et al.* (2010), prefixed with RC, and sequences from GenBank with other identifiers. In-group taxon names are colour coded according to geographic occurrence.

TABLE 3. Component loadings from Principal Component Analysis of morphometric data from *D. absconditus* (N=12), *Danio annulosus* (N = 10), *D. assamila* (N=6), *D. catenatus* (N=15), *D. concatenatus* (N=9), *Danio dangila* (N=9), D. *feegradei* (N=17), *D. meghalayensis* (N=5) and *D. sysphigmatus* (N=11). The two highest loadings for each component II-IV are highlighted in boldface.

	PC I	PC II	Sheared PC II	PC III	Sheared PC III	PC IV	Sheared PC IV
Standard length	0.231	-0.147	-0.120	0.188	0.19	-0.007	-0.014
Body depth	0.249	-0.005	0.022	-0.234	-0.232	0.438	0.432
Head length	0.195	-0.073	-0.052	0.025	0.026	-0.056	-0.062
Snout length	0.225	-0.015	0.009	0.131	0.133	-0.305	-0.312
Head depth	0.182	-0.147	-0.126	-0.222	-0.221	0.178	0.173
Head width	0.214	-0.164	-0.139	-0.026	-0.024	-0.096	-0.102
Upper jaw length	0.180	-0.102	-0.082	0.044	0.046	-0.123	-0.128
Lower jaw length	0.178	-0.086	-0.066	0.077	0.079	-0.217	-0.222
Orbital diameter	0.143	0.001	0.017	-0.142	-0.141	0.272	0.269
Interorbital width	0.226	0.013	0.037	-0.002	0.000	-0.092	-0.098
Caudal peduncle length	0.148	-0.570	-0.547	-0.094	-0.093	-0.162	-0.167
Caudal peduncle depth	0.245	-0.239	-0.210	-0.104	-0.102	0.203	0.196
Dorsal-fin base length	0.337	**0.465**	**0.496**	**0.384**	**0.387**	-0.250	-0.259
Anal-fin base length	0.278	0.216	0.244	0.364	0.366	**0.589**	**0.582**
Predorsal length	0.226	-0.165	-0.139	0.168	0.17	-0.009	-0.015
Preanal length	0.242	-0.126	-0.099	0.126	0.128	-0.048	-0.054
Prepelvic length	0.231	-0.127	-0.100	0.072	0.074	-0.034	-0.04
Pectoral-fin length	0.253	**0.355**	**0.378**	-0.312	-0.31	-0.145	-0.152
Pelvic-fin length	0.285	0.277	0.305	**-0.606**	**-0.604**	-0.156	-0.164
Eigenvalue	0.955	0.028	N/A	0.010	N/A	0.008	N/A
Cumulative variance (%)	93.6%	96.3%	N/A	97.3%	N/A	98.0%	N/A

Genetic distance has been used as an aid in delimiting putative species in numerous studies. Hebert *et al.* (2003b) and Ward (2009) empirically found that if the corrected pairwise *p*-distance in COI between two individuals of fish is ≥2% they are likely (≥95%) to be of different species, while if their pairwise p-distance is less than 0.4% they are likely (>89%) to be conspecific.The "barcoding gap" between intra- and interspecific variation varies in size and can be indistinct. Hebert *et al.* (2004) suggested using Kimura-2-parameter corrected intraspecific *p*-distance multiplied by ten as a threshold for species delimitation. The intraspecific *p*-distance in *D. annulosus* is 0, and the closest *p*-distance to the most similar included species, *D. catenatus*, is 3.4%.The intraspecific corrected *p*-distance of *D. annulosus* is 0.2% ("corrected" distance = an evolutionary model has been used to compensate for hypothetical back mutations; therefore corrected distance is greater than zero even though all three *D. annulosus* sequences are identical). Corrected *p*-distance between *D. annulosus* and its closest relative, *D. catenatus*, is 4.0%, or 20 times greater than the corrected intraspecific distance.

Monophyletic groups in gene trees can be artefacts caused by stochastic gene coalescence within a larger panmictic group. Rosenberg's P_{AB} statistic (Rosenberg, 2007) is an estimate of the probability that a putative species is monophyletic relative to its closest sister clade under a null model of random coalescence. Rosenberg's P_{AB} for *D. annulosus* is 0.17, indicating a low probability that it is an artefact caused by stochastic coalescence. Robinson *et al.* (2009) found that the ratio between intra- and interspecific distances outperformed arbitrary fixed distance thresholds in indicating species. Masters *et al.* (2011) expanded on this by proposing P ID(*Liberal*), a statistical comparison of the distribution of intraspecific variation within a putative species to the interspecific distance and intraspecific variation within its closest relative, which estimates the mean (95% confidence interval) probability of correctly identifying an unknown member of the putative species, with the criterion that it should fall within or sister to the putative species. P ID(*Liberal*) for *D. annulosus* is 0.98, indicating a high probability that an unknown member of the species would be identified as a member of the putative species.

Collins *et al.* (2012) produced COI and Rhodopsin sequences for many species of *Danio*, including several chain danio species, in a study demonstrating a standard library for combating biohazards posed by aquarium imports into New Zealand. The ease with which the wrong sequences could be uploaded as barcodes, in combination with the unspecific identification, suggests that aquarium fishes and imprecise identifications by non-specialists may not be the best approach for building a DNA barcode reference library. By making the holotype of *D. annulosus* also the barcode voucher for the species, we create a very strong link between the taxonomy and the barcode in this case. It is rarely possible to do this, however, because the fixation for enabling continued DNA harvesting from the voucher (frozen, dried, in ethanol) affects the quality of the specimen, whereas the preferred fixation in formalin makes DNA extraction difficult or impossible. In the present case the holotype is in good condition even after ethanol preservation, and the linkage between taxonomy and DNA barcode was given priority.

The phylogram (Fig. 7) suggests that *D. annulosus* is the sister group of *D. catenatus* from western Myanmar, and these two species make up the sister group of *D. concatenatus* from western Myanmar and *D. sysphigmatus* from eastern Myanmar. This group in turn is the sister group of the mainly Indian *D. dangila,* and Brahmaputra species *D. assamila* and *D. meghalayensis.* The tree may indicate a deep split between species in the Ganga-Brahmaputra basin and those in south-western Bangladesh and Myanmar. A significant part of the Bangladeshi fish fauna is shared with India in the Ganga and Brahmaputra basins (e.g., Siddiqui *et al.* 2007) and is distinct from the Myanmar-Indochina fauna. Eastern Bangladesh is a mountain region that is part of the Indo-Myanmar Ranges. The close relationship of *D. annulosus* to *D. catenatus* south and west of the Kaladan drainage, as well as the similarity and close relationship of *Devario anomalus* Conway, Mayden & Tang from Cox's Bazar, and *D. xyrops* Fang & Kullander from western Rakhine State (Fang & Kullander, 2009) suggests a distinct fauna from that of the Ganga basin. Coverage of collections in the relevant regions of Bangladesh and Myanmar is still fragmentary, but the distribution of chain danios and *D. xyrops*+*D. anomalus* suggests that the Rakhine Yoma hotspot may extend into Bangladesh as a succession of species along the southern hills of the Indo-Burma ranges.

The Shuvolong Falls, type locality of *D. annulosus*, are spectacular and represent also a popular tourist target. The falls are a series of relatively concentrated free cascades flowing from a steep sandstone rock down to a small terrace about 90 m below. During the rainy season a large pool forms on the terrace, and water exits to the lake through a small stream. During the dry season only little water trickles down the hill. The terrace is then exposed with only at most knee deep water remaining in the shadowed, slightly deeper part, and the effluent stream all but disappears. It was not possible to ascend the hill from which the water flows to examine a possible stream feeding the waterfall, but it seems unlikely that the *Danio* originate from the waterfall. The pool at the bottom of the major fall may dry up completely in the extreme dry season, and we consider it unlikely that it harbours permanent populations of the two fish species collected there. It seems more likely that the *Danio* and *Garra* are attracted by the running water during the rainy season, and get captured in the pool as the lake level sinks, exposing the platform at the base of the hill. Most likely there was before the filling of the lake a longer stream from the fall which harboured a permanent population of *D. annulosus* and the syntopic *Garra*. There are two possible explanations for the replenishment of the habitat. Either there are small, still unexplored streams in the adjacent hills holding permanent populations which expand to reach the base of the falls during the wet season, or, more likely, a significant part of the populations survive in lake conditions close to the falls. Most of the hills in the Kaptai Lake region are still ichthyologically unexplored, and it seems reasonable to expect that both the *Garra* and *D. annulosus* have wider distributions.

Danio dangila has been reported from Bangladesh by Barman (1991) and Rahman & Chowdhury (2007), and specifically from near Cox's Bazar by Rahman (1989, 2005) and Ahmed *et al.* (2013), and from the Halda River near Chittagong by Alam *et al.* (2013). The Halda is a tributary of the Karnafuli River close to its mouth. Our sampling in the lower Halda River drainage in 2014 did not yield any *D. dangila*, and we have not been able to examine specimens from Cox's Bazar. Ahmed & Hasan (1981) did not find any *Danio* in their inventory of the Kaptai Lake fish fauna, but their efforts were restricted to fishing vessels, markets, and the Rangamati fish landing. We also examined such sources in and near Rangamati in November 2014, but no *Danio* or other small stream fish species were present in the commercial catches.

"*Danio dangila*" in past literature is a catch-all name for several species with a particular colour pattern. Based on the present report and the revision by Kullander (2015), this may represent not only a remarkably widespread monophyletic group of danios occurring over a large part of India, Nepal, Bangladesh, and Myanmar, but also cryptic species with about the same shape and colour pattern, which unambiguously are distinct species based on DNA differences. This species assemblage may have acquired its diversity by fragmentation in isolated cooler mountain streams separated by warm lowland rivers. Kullander (2015) suggested that cooler and drier lowlands during glacial periods may have provided habitats amenable to dispersal, followed by isolation and speciation during interglacials like the present. The continued discovery of new species of chain danios, however, also suggests that we are far from a complete inventory of the species of this group.

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